PURIFICATION, CHARACTERIZATION AND COMPARISON WITH MAMMALIAN SCP_2 OF A CHICKEN SCP_2-LIKE PROTEIN

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Abstract—1. Three proteins have been isolated from chicken (*Gallus domesticus*) liver that bind antibodies directed against authentic rat sterol carrier protein$_2$ (SCP_2) and have similar molecular mass to the three major immunoreactive rat liver proteins (12 kDa, 30–36 kDa, 55–60 kDa).

2. Bile from both chicken and rat contains the high molecular mass immunoreactive species.

3. The chicken 12 kDa SCP_2-like protein purifies similarly to rat SCP_2 but the homogeneous chicken SCP_2-like protein is dissimilar in amino acid composition and *N*-terminal amino acid sequence.

4. The activity of chicken SCP_2-like protein differs from rat SCP_2 in that it was consistent with fusion (transfer of both polar surface and non-polar core lipids) rather than transfer of polar lipids only.

INTRODUCTION

Movement of lipids within cells can result from diffusion through the cytoplasm, membrane movement, carrier-mediated transport or a combination of these (Reinhart, 1990; Billheimer and Reinhart, 1990). Several proteins have been implicated in the specific transfer of phospholipids between membranes in support of carrier-mediated transport (Wirtz and Gadella, 1990). A non-specific lipid transfer protein (nsLTP) has also been identified and purified from mammalian sources (Bloj and Zilversmit, 1977; Trzaskos and Gaylor, 1983). This protein is known as SCP_2, since it transfers cholesterol between donor and acceptor membranes in *in vitro* assays; it is also capable of transferring many polar lipids by an undefined mechanism (Wirtz and Gadella, 1990). This protein can stimulate many of the reactions of cholesterol biosynthesis (Noland *et al*., 1980; Trzaskos and Gaylor, 1983) and could be involved in the synthesis of other lipids. These functions make SCP_2 an important component of lipid synthesis and mobilization pathways.

Little is known about the presence of non-specific lipid transfer activity in avian systems. Initial identification of several lipid transfer proteins in chicken liver was recently described (Rusinol and Bloj, 1989). Our interest was in determining whether these activities resulted from an SCP_2-like protein. Since no other avian SCP_2 protein has been purified, we decided to compare it to mammalian SCP_2. In this paper, we describe the purification of a chicken protein which is of similar molecular mass and antigenic make-up to the mammalian SCP_2, but which differs significantly in amino acid composition and *N*-terminal sequence. This is reflected in its altered activity.

‡Author to whom correspondence should be addressed. Mention of brand or firm names does not constitute an endorsement by the US Department of Agriculture over others of a similar nature not mentioned.

MATERIALS AND METHODS

Purification of liver fractions

Broiler chickens (2.2–3.6 kg) were used throughout this study. A modification of the method of Trzaskos and Gaylor (1983) was employed for the purification of the SCP_2-like protein. Chickens were sacrificed by cervical dislocation. Livers were excised, weighed, and homogenized with a Potter–Elvehjem homogenizer fitted with a Teflon pestle in 3 vols of 1.0 mM Tris–HCl buffer, pH 7.5, containing 250 mM sucrose, 0.5 mM dithiothreitol (DTT) and 20 μM leupeptin. The homogenate was centrifuged at 1000 g for 10 min, and the supernatant removed and centrifuged at 15,000 g for an additional 20 min. This supernatant (S15) was used as starting material for the purification of the SCP_2-like protein. For some experiments the S15 was centrifuged at 105,000 g for 1 hr to separate microsomal and soluble components (S105). All steps after removal of the livers were performed at 4°C unless otherwise noted.

Purification of the SCP_2-like protein

The S15 was adjusted to pH 5.1 with 5 N HCl and stirred for 30 min. The mixture was centrifuged for 20 min at 16,300 g, and the supernatant was titrated to pH 7.5 with 5 N NaOH prior to addition of ammonium sulfate to 40% saturation. Following stirring for 45 min, the mixture was centrifuged (16,300 g for 20 min) and the precipitate removed. The supernatant was adjusted to 80% saturation with ammonium sulfate and stirred for 45 min. The 40–80% ammonium sulfate precipitate was collected by centrifugation and resuspended in 15 ml of 10 mM potassium phosphate buffer, pH 6.8, containing 1.0 mM EDTA and 1.0 mM DTT (KPED buffer). The sample was placed into dialysis tubing (Spectrapor 23 mm tubing, 6–8000 M.W.C.O.) and dialyzed overnight against 2–150 vol portions of KPED buffer. The dialyzed sample was then subjected to ultrafiltration with a Diaflo PM30 membrane (Amicon Corp.), and the filtrate concentrated by a Diaflo

YM10 membrane. This concentrate is subsequently referred to as the YM10 retentate.

Immunoaffinity purification

The YM10 retentate was dialyzed overnight against 2–600 vol portions of 10 mM Tris–HCl buffer, pH 8.1, containing 140 mM NaCl and 0.02% NaN_3 (wash buffer) and applied to an immunoaffinity column (kindly donated by Dr. Jeffrey T. Billheimer) containing purified rabbit anti-rat SCP_2 conjugated to CNBr activated Sepharose 4B. Unbound protein was eluted with the wash buffer following a 16 hr incubation. Bound protein was eluted by batch method using 100 mM sodium citrate buffer, pH 3.0, containing 0.02% NaN_3. The acid-eluted fractions were adjusted to pH 7.4 with Tris–base, dialyzed overnight using 2–70 vol portions of 50 mM ammonium acetate, and lyophilized.

Protein determination

Protein concentrations were determined by the method of Bradford (1976) using bovine γ-globulin as standard.

Amino acid analysis

The amino acid content of the homogeneous immunopurified protein was determined by hydrolysis with 5.7 N HCl, containing phenol (0.05%), in sealed, evacuated tubes at 110°C for 24 hr. Analysis was performed using a Beckman 119 Cl amino acid analyzer (Greenberg *et al.*, 1984). *N*-Terminal amino acid sequencing was by automatic Edman degradation technology on a Porton model PI2090 gas phase sequencer (Hunkapiller *et al.*, 1983).

Transfer assay

The unidirectional transfer of cholesterol was measured in a liposomal/mitochondrial assay system according to a slight modification of the method of Billheimer and Gaylor (1990). Liposomes containing [^{14}C] cholesterol (1 Ci/mol), [^{3}H] triolein, and unlabeled egg phosphatidylcholine were prepared in 20 mM Tris–HCl buffer, pH 7.4, containing 250 mM sucrose, 1 mM EDTA and 0.002% NaN_3 (SET buffer). To measure cholesterol transfer, 3 mg unlabeled heat-inactivated rat liver mitochondria, 1 mg bovine serum albumin, 2–200 μg protein and liposomes were mixed and adjusted to 0.5 ml with SET buffer. The reaction mixture was incubated for 20 min at 37°C with shaking. Transfer was terminated by placing the reaction mixture on ice followed by centrifuging (15,000 *g* for 20 min) to remove the mitochondria. The ratio of [^{3}H] to [^{14}C] radioactivity remaining in the supernatant was quantified by liquid scintillation spectrometry and the per cent of transfer was calculated and corrected for the amount of transfer in control assays which contained no SCP_2, (approximately 10%).

Polyacrylamide gel electrophoresis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed in the presence of 5% 2-mercaptoethanol, according to the method of Laemmli using a 12% resolving gel with 0.1% SDS (pH 8.8) and a 3% stacking gel with 0.1% SDS (pH 6.8). Molecular masses of polypeptides were determined by comparison with standard proteins. Gels were stained with Coomassie Brilliant Blue R-250 or silver stained (Bio-Rad). Homogeneous 20% Phastgels (Pharmacia) were also used for SDS–PAGE and Western blotting.

Western blots

Samples were electrophoresed on 12% SDS–PAGE gels as described above. The samples were transferred to nitrocellulose (Burnette, 1981) and probed with rabbit anti-rat SCP_2 antiserum. Bound antibody was detected using either horseradish peroxidase or alkaline phosphatase conjugated goat anti-rabbit IgG (GAR–HRP or GAR–AP, Bio-Rad).

RESULTS

In order to determine whether an SCP_2-like protein is present in chicken liver, and to compare it with authentic mammalian SCP_2, whole homogenates and an S105 fraction were prepared from both rat and chicken livers. In addition, bile was collected from rat by cannulation and from the chicken by withdrawal directly from the gall bladder. Aliquots of each sample were electrophoresed on 12% SDS–PAGE gels, Western blotted, and probed using rabbit anti-rat SCP_2 antiserum. Antibody labeled proteins were localized using GAR–HRP (Fig. 1). Several distinct proteins were revealed by this procedure. Three major proteins were present in the rat whole homogenate and S105, and proteins of similar molecular mass were seen in analogous chicken liver fractions. Each of these species has been observed previously using this antiserum and by other investigators using independently produced antibodies (Van Amerongen *et al.*, 1987; Tsuneoka *et al.*, 1988). To our knowledge, bile has not previously been examined for the presence of SCP_2 in any species. In both the chicken and the rat only the highest molecular mass immunoreactive species was present in the bile.

Since the three major immunoreactive proteins in the chicken appear to be similar (in molecular mass and epitope composition), it was presumed that the 12 kDa form might be purified using the protocol developed for the mammalian SCP_2. Therefore, the procedure of Trzaskos and Gaylor (1983) was modified for the chicken, as described in Materials and Methods. Fractions were electrophoresed on 12% SDS–PAGE gels and were either stained for protein using Coomassie Blue (Fig. 2A) or Western blotted (Fig. 2B). Western blotted proteins were

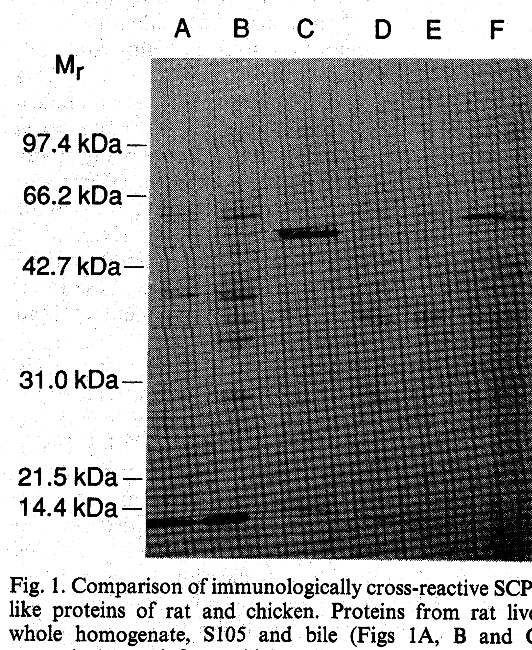

Fig. 1. Comparison of immunologically cross-reactive SCP_2-like proteins of rat and chicken. Proteins from rat liver whole homogenate, S105 and bile (Figs 1A, B and C, respectively) and from chicken liver whole homogenate, S105 and bile (Figs 1D, E and F, respectively) were electrophoresed on 12% SDS–polyacrylamide gels and electroblotted on to 0.2 micron pore size nitrocellulose. Blotted proteins were probed with rabbit anti-rat SCP_2 antiserum and bound antibodies were visualized using GAR–HRP.

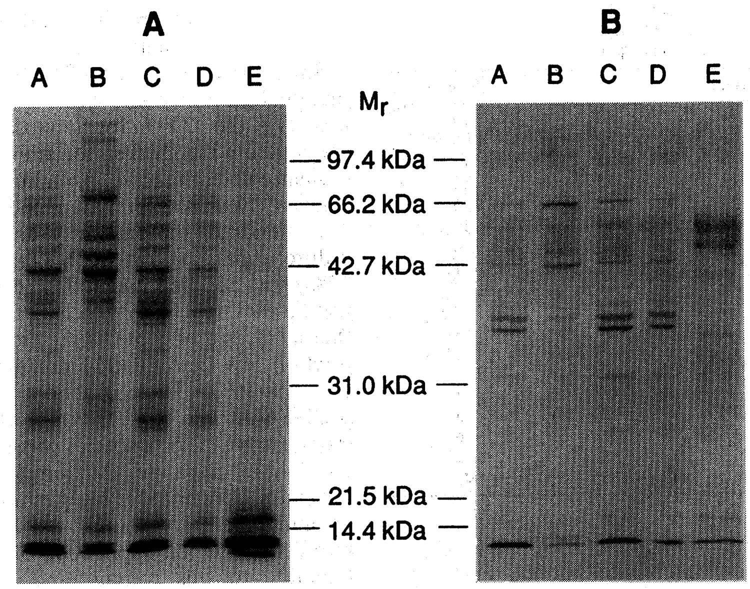

Fig. 2. SDS–PAGE and Western Blot analysis of purification fractions. Aliquots obtained during the purification of the 12 kDa SCP$_2$-like protein were treated with SDS/mercaptoethanol and separated on 12% acrylamide gels. One gel was stained with Coomassie Blue (Fig. 2A) while an identical gel was Western blotted, probed with rabbit anti-rat SCP$_2$ antiserum, and the bound antibodies visualized with GAR–AP (Fig. 2B). Lanes contain: A, acid treated supernatant; B, 40% ammonium sulfate pellet; C, 40–80% ammonium sulfate pellet; D, PM30 retentate; and E, YM10 retentate.

probed as in Fig. 1. The 12 kDa chicken SCP$_2$-like protein was purified along the lines followed by rat SCP$_2$ and was recovered free of higher molecular mass immunoreactive species in the YM10 retentate fraction (Fig. 2B, lane E).

In the YM10 retentate fraction applied to an immunoaffinity column, the majority of protein in the YM10 retentate was not adsorbed to the immunoaffinity support. Specifically bound protein was eluted using a sodium citrate buffer of pH 3.0, dialyzed and freeze-dried. The eluted material was electrophoresed on 12% SDS–PAGE and stained (Fig. 3A) or was Western blotted, probed with rabbit anti-rat SCP$_2$ antibodies and visualized using GAR–AP (Fig. 3B). A single polypeptide of 12 kDa was observed by staining and this protein was recognized by rabbit anti-rat SCP$_2$ IgG in Western blotting procedures. Amino acid analysis was performed on the chicken protein to compare the avian and mammalian forms of the immuno-crossreactive proteins (Table 1). The chicken protein varies considerably from the mammalian SCP$_2$ in amino acid composition. To determine if similarities in sequence were present in the amino terminus, sequencing of the chicken protein was performed (Fig. 4). No sequence similarity was observed within the *N*-terminal region.

Because of these differences in amino acid composition and sequence between SCP$_2$ and the avian SCP$_2$-like protein, the *in vitro* activity of the protein was assayed. Non-specific lipid transfer activities of various purification fractions were determined in an *in vitro* assay (Table 2). While a non-specific lipid transfer activity is present during early stages of purification, it is not found in the purified protein. The highest spec. act. was actually observed in the fraction of proteins with molecular mass greater than 30 kDa. While transfer activity was not observed in purified preparations of the avian SCP$_2$-like protein, fusion activity (i.e. the total assimilation of both polar ([^{14}C] cholesterol) and non-polar ([^{3}H] triglyceride) lipid fractions of donor particles to acceptor particles) was observed in a number of preparations.

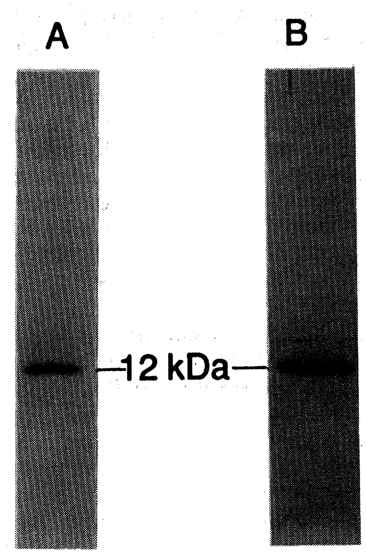

Fig. 3. Affinity purification of chicken SCP$_2$-like protein. The YM10 retentate fraction was dialyzed and applied to a 5 ml column of CNBr-activated Sepharose 4B containing immobilized rabbit anti-rat SCP$_2$ IgG. A large peak of unabsorbed protein was washed from the column before the elution of bound material by reduction of pH to 3.0 with a citrate buffer. Eluted protein was neutralized, dialyzed with ammonium acetate buffer and lyophilized. SDS–PAGE separation and silver staining (A) revealed a single polypeptide of 12 kDa. Western blot (B) analysis confirmed that this polypeptide binds the anti-SCP$_2$ antibodies.

Table 1. Amino acid composition* of chicken SCP_2-like protein with authentic bovine (Westerman and Wirtz, 1985) and rat (Pastuszyn *et al.*, 1987) SCP_2

	Bovine	Rat	Chicken
Asp(n)	14	13	10
Thr	3	2	6
Ser	5	7	13
Glu(n)	12	13	15
Pro	4	3	6
Gly	13	14	16
Ala	9	10	6
Cys	1	1	1
Val	8	6	7
Met	4	4	2
Ile	5	4	4
Leu	12	9	8
Tyr	ND	ND	5
Phe	7	7	3
His	ND	ND	3
Lys	19	19	6
Arg	ND	ND	4
Trp	ND	ND	ND

*Number of residues.
ND: not detected.

DISCUSSION

Rusinol and Bloj (1989) demonstrated the presence in chicken liver of several lipid transfer activities, some of which were responsive to hormone treatment and were regulated in ways which suggested involvement in lipid synthesis and mobilization (i.e. lipoprotein assembly). Since SCP_2 has been implicated in mammalian cells in both transfer activity and modulation of lipid synthetic activity, we questioned whether any of the activities described by these authors could be accounted for by an avian SCP_2-like molecule. In preliminary studies, we reasoned that such a protein might be conserved enough in evolution that antibodies directed against the mammalian protein would recognize the avian form. A highly-specific antiserum directed against rat SCP_2 recognized three major proteins in both rat and chicken homogenates. Interestingly, the three proteins in the two species were in similar molecular mass ranges. Evidence currently available suggests that these three forms in mammals might in fact arise by alternate processing of a single gene product (Billheimer *et al.*, 1990) and that at least one higher molecular mass form contains the entire sequence of the lowest molecular mass form (Billheimer *et al.*, 1990). The chicken, unlike the rat, stores bile in a gall bladder. We investigated to see if an SCP_2-like molecule was present in this fluid. Figure 1 shows clearly that a high molecular mass (55–60 kDa) protein cross-reactive with anti-SCP_2 antibodies was present. This prompted us to collect bile from rats by cannulation and to probe for SCP_2 using antibodies. Again, a high molecular mass form (but neither of the lower two major molecular mass forms) was observed.

Since the only species of SCP_2-like protein in the mammal currently known to have authentic nsLTP activity is the 12 kDa form, we decided to purify this species from the chicken for amino acid analysis and sequence determination. An homogenate of four 50 g chicken livers was prepared and from this a post-mitochondrial supernatant was obtained. A procedure applied to rat SCP_2 (Trzaskos and Gaylor, 1983) was adopted and followed with ultrafiltration membranes replacing a gel filtration column, such that a fraction of proteins greater than 30 kDa and a fraction less than 30 kDa but greater than 10 kDa was obtained. These fractions were analyzed by SDS–PAGE and by Western blotting. The immuno-crossreactive 12 kDa species was recovered mainly in the YM10 retentate fraction (Fig. 2). Since this protein was identifiable by Western blotting, we used an immunoaffinity column as the final step in the purification. Upon application of a sample of YM10 retentate to this column and following extensive washing, an homogeneous protein band was eluted with citrate buffer, pH 3.0. This band was 12 kDa by SDS–PAGE and proved positive by Western blotting.

Amino acid analyses of this protein (Table 1) showed a great dissimilarity to authentic rat and bovine SCP_2s [which show greater than 90% homology with each other (Westerman and Wirtz, 1985; Pastuszyn *et al.*, 1987; Morris *et al.*, 1988)]. Especially obvious is the lack of arginine and histidine in mammalian SCP_2, but their presence in the avian protein. This prompted us to examine the *N*-terminal sequence for possible homology in that section (Fig. 4). Again dissimilarity was observed between the avian and mammalian proteins while the two mammalian proteins were quite similar.

Because of the differences in amino acid composition, we decided to set up an SCP_2 *in vitro* transfer assay using a modification of the system of Trzaskos and Gaylor (1983). Surprisingly, the avian SCP_2-like protein did not show true transfer activity in this assay system. In several preparations, however, fusion activity was observed, indicating that the protein function is also somewhat altered. An examination of SCP_2 assays of other purification fractions revealed that true SCP_2-like activity is present and co-purifies with the 12 kDa form during the early stages of purification but does not pass through the PM30 membrane as shown in Table 2. This indicates that it is either in a multimeric form too large to pass through a filter or is of molecular mass greater than 30 kDa. Western blotting of this fraction has demonstrated the presence of a 36 kDa immuno-crossreac-

RAT	Ser	---	Ser	Ala	Gly	Asp	Gly	Phe	Lys	Ala	Asn	Leu	Ile
	●		●			●	●	●	●	●	●	●	
BOVINE	Ser	---	Ser	Ser	Val	Asp	Gly	Phe	Lys	Ala	Asn	Leu	Val
CHICKEN	Glu(n)		Thr	Pro	Leu	Tyr	Pro	Lys	Val	Tyr	Thr	Met	Asp

Fig. 4. *N*-Terminal sequence comparison. The *N*-terminal amino acid sequence was determined for the immunoaffinity purified chicken protein. Here, it is compared with the sequences derived for the bovine (Westerman and Wirtz, 1985; Morris *et al.*, 1988) and rat (Pastuszyn *et al.*, 1987) SCP_2 molecules (● denotes common amino acids). While the mammalian proteins show a high degree of sequence similarity, the chicken protein shows no similarity.

Table 2. Transfer activity of purification fractions of chicken SCP_2-like protein

Acid treated supernatant	40–80% Pellet	PM30 retentate	YM10 retentate	Affinity pure
20%*	28%	48%	13%	<1%

*Per cent lipid transfer/mg protein.

tive protein (Figs 1, 2). Further purification of this material (currently to three protein bands) demonstrates that the SCP_2 activity is in a fraction which does contain the immunoreactive band. Currently we are attempting to determine whether this protein represents the true avian SCP_2. If it does, it will be of great interest to determine whether the higher molecular mass forms of mammalian SCP_2 also have true SCP_2 activity.

Several interesting questions are posed by this study. First, where within the avian SCP_2-like molecule does the common epitope(s) lie and how extensive is it? To answer this question, more extensive sequencing is underway. Secondly, do such regions of homology lie within the proposed amphipathic helix regions (Pastuszyn *et al.*, 1987) or other regions of the protein? An important question to be addressed in the mammalian system based on our study is whether the mammalian higher molecular mass forms also have SCP_2 activity. This may have implication in humans, where in the Zellweger's Syndrome (Cerebro–hepato–renal syndrome) the 12 kDa and 55 kDa proteins are missing in liver cells (Van Amerongen *et al.*, 1987). We have recently found that a 30–36 kDa form of immuno-crossreactive protein is present in cultured fibroblasts from patients with Zellweger's Syndrome (Johnson and Reinhart, unpublished results). Some cells of Zellweger's Syndrome patients might therefore contain SCP_2 activity.

Finally, the reason for a high molecular mass SCP_2-like species in the bile is of interest. A related protein (55 kDa SCP_2-like protein) has been demonstrated to be restricted to peroxisomes (Van Amerongen *et al.*, 1987; Keller *et al.*, 1989). Thompson *et al.* (1987) and Appelkvist (1987) have shown that cholesterol synthesis proceeds in isolated peroxisomes in the presence of cytoplasm. In addition, Appelkvist *et al.* (1990) have demonstrated cholesterol 7-α-hydroxylase activity in the peroxisome while others (Hagey and Krisans, 1982; Thompson and Krisans, 1985) have demonstrated the presence of additional enzymes that participate in the synthesis of bile acids. Perhaps bile acids synthesized in peroxisomes are somehow tied to the SCP_2-like molecule and are expelled into the bile together. Such a molecule might function in the intestine by transferring lipids across intestinal cell membranes or by doing so after conversion to a lower molecular mass form. Such questions are the focus of our current studies.

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